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PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Small Entity)

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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TITLE OF THE INVENTION (280 characters max)

Characterization of a Novel Receptor Kinase From Brassica with a Putative Role in Plant Defense

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Respectfully submitted,

SIGNATURE

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CHARACTERIZATION OF A NOVEL RECEPTOR KINASE FROM BRASSICA WITH A PUTATIVE ROLE IN PLANT DEFENSE

FIELD OF THE INVENTION

The present invention is directed to the characterization of a gene from *Brassica napus* the transcription of which is induced by wounding or chemical elicitors that mimic a pathogen attack. More particularly, the present invention is directed to the isolation of a cDNA clone designated PERK1 (Proline Extensin-like Receptor Kinase 1) which is proposed to encode a putative novel receptor kinase in *Brassica napus* and comprises a new class of plant receptor kinases characterized by an extracellular domain rich in proline and sharing sequence similarity to the extensin family of cell wall proteins.

BACKGROUND OF THE INVENTION

Receptor mediated signal perception and transduction in response to external stimuli are essential for growth and developmental processes of multicellular organisms (Mu et al., 1994). These extensively well characterized processes in animal systems involve receptor protein kinase molecules comprised of an extracellular signal perception domain, a hydrophobic transmembrane domain attached to an intracellular domain that possesses kinase activity (Horn et al., 1994). In general, transmembrane signaling by receptor protein kinases requires binding of an appropriate ligand to the extracellular domain which induces receptor dimerization and alters the activity of the intracellular catalytic domain. This promotes phosphorylation of specific substrates thereby initiating a protein kinase signaling cascade (Ullrich and Schlessinger, 1990). The majority of animal receptor protein kinases isolated to date contain tyrosine-specific kinase domains (Ullrich and Schlessinger, 1990), however, the transforming growth factor β (TGF- β) receptor (Lin et al., 1992) and the activin receptor (Dijke et al., 1993) possess kinase domains with serine/threonine phosphorylation activity.

Intracellular communication is also essential for the growth and development of higher plants. The extensive knowledge of cell surface receptor signaling in animal systems has resulted in the isolation of several genes predicted to encode putative receptor-like protein kinases (RLKs). The characterized members of the RLK family share highly homologous

catalytic domains with consensus sequences indicative of serine/threonine autophosphorylation activity, yet the extracellular domains of these receptors are very divergent (Braun and Walker, 1996). Five different classes of plant receptor-like protein kinases have therefore been identified according to amino acid sequence similarity in the extracellular domains of these genes. The first class of receptor kinases, designated the S-domain class, have distinct extracellular domains homologous to the S-locus glycoprotein (SLG) (Nasrallah and Nasrallah, 1993). S-domain receptor kinases have several distinguishing features such as ten conserved cysteine residues located proximal to the transmembrane domain in addition to other conserved residues implicated in the proper folding of the extracellular domain (Walker, 1994). Among this class of receptor kinases are the S-locus receptor kinases (SRKs) of *Brassica* expressed exclusively in reproductive tissues and implicated along with SLGs in controlling the sporophytic self-incompatibility response which normally inhibits self-pollination (US Patent 5,821,094; US Patent 5,484,905; Stein et al., 1991; Goring and Rothstein, 1992). Other receptor-like kinases of this type are represented in *Arabidopsis* by ARK1, ARK2, ARK3 (Tobias et al., 1992; Dwyer et al., 1994), in maize by ZmPK1 (Walker and Zhang, 1990) and by OsPK10 in rice (Zhao et al., 1994). The diversity in patterns of expression among members of the S-domain class suggests that these putative plant receptor kinases may be involved in mediating a variety of cellular signaling processes (Walker, 1994).

Another class of plant receptor kinases is the leucine-rich repeat (LRR) group which encodes proteins with extracellular domains containing 20-25 imperfect repeats of a 24 amino acid leucine-rich motif involved in peptide ligand recognition, cell adhesion and implicated in mediating protein-protein interactions (Braun and Walker, 1996; Wang et al., 1998). This class of plant receptor kinases include proteins such as CLAVATA1 (Clark et al., 1997) which is involved in regulating meristem and flower development in *Arabidopsis*, as well as proteins functioning in gamete development such as PRK1 of *Petunia* (Mu et al., 1994). The LRR class is represented in *Arabidopsis* by other receptor kinases such as ERECTA (Torii et al., 1996) which has been shown to be essential for proper plant and organ elongation, BRI1, a receptor involved in brassinosteroid signal transduction (Li and Chory, 1997), as well as TMK1 (Chang et al., 1992) and RLK5 (Walker, 1993) which may have more general roles in cellular signaling as suggested by their ubiquitous expression patterns in a variety of vegetative and reproductive

tissues. Xa21, another member of the LRR class, has been implicated in pathogen recognition by providing resistance in rice to *Xanthomonas oryzae* pv. *oryzae* (Song et al., 1995).

The lectin-like class of plant receptor kinases is represented only in *Arabidopsis thaliana* by Ath.lecRK1 (Hervé et al., 1996) and LRK1 (Swarup et al., 1996). The extracellular domain of these receptor kinases share sequence similarity with lectins which are known carbohydrate binding proteins and implicated in the transduction of oligosaccharide signals in plant cellular communication processes (Hervé et al., 1996).

The two remaining classes of plant receptor kinases isolated in *Arabidopsis thaliana* include proteins with extracellular domains containing epidermal growth factor (EGF)-like motifs found in many proteins involved in extracellular interactions (WAK1; Kohorn et al., 1992) as well as thaumatin-like domains homologous to PR5 proteins involved in pathogenesis (PR5K; Wang et al., 1996).

Extensins are particularly abundant proteins in plant cell walls and are very rich in proline and serine as well as in combinations of valine, tyrosine, lysine and/or histidine residues. The distinctive characteristic of dicot extensins is their repetitive (Ser-Pro)₄ pentapeptide blocks. Although extensins are synthesized as soluble precursors, the majority of proline residues are hydroxylated and both the hydroxylated proline as well as the serine residues of these proteins are glycosylated by post-translational modifications (Cassab, 1998). When secreted to the plant cell wall, extensins become rapidly insoluble, presumably due to the formation of covalent isodityrosine bridges (Cassab, 1998). Although extensins have been proposed to be structural cell wall proteins and important in development, they have also been directly implicated in plant defense against mechanical wounding (Shirsat et al., 1996) and pathogen attack (Corbin et al., 1987; Showalter, 1993).

Many of the inducible defense responses are not exclusive to mechanical wounding but are also initiated by pathogen attack. The similarity between responses to wounding and pathogen attack are not surprising since mechanical damage often precedes pathogen infection and conversely, mechanical damage may often result from a pathogen or insect attack (Truernit et al., 1996). Salicylic acid has been implicated in having an important role in the signal transduction pathway leading to systemic acquired resistance (SAR) (Penninckx et al., 1996). In general, plants challenged by mechanical wounding or pathogen attack induce rapid expression of genes (ie. proteinase inhibitor (*pin*) and pathogenesis related (*PR*) genes respectively) that are

expressed locally as well as systemically in unaffected parts of the plant (Yang et al., 1997). Increased levels of extensin transcripts as a result of mechanical wounding have been well established in various plants (Sauer et al., 1990; Shirsat et al., 1996).

It is an object of the present invention to isolate from the cDNA library of *Brassica napus* a receptor-like protein kinase designated PERK1 (Proline Extensin-like Receptor Kinase) which represents a new class of plant receptor kinases characterized by an extracellular domain rich in proline and sharing sequence similarity to the extensin family of cell wall proteins. It is postulated that PERK1 may be involved in the general perception and subsequent transduction of a wound and/or pathogen stimulus, ultimately triggering a plant's defense mechanisms and conferring broad protection against such stimuli.

SUMMARY OF THE INVENTION

In general, the present invention provides the characterization of an isolated DNA molecule comprising the nucleic acid sequence of SEQ ID No 1. In another embodiment, the present invention provides the characterization of an isolated DNA molecule having at least 30% homology to the nucleic acid sequence of SEQ ID No 1. In yet another embodiment, the present invention provides the characterization of an amino acid molecule having the amino acid sequence of SEQ ID No 2. In yet another embodiment, the present invention provides the characterization of an isolated DNA molecule coding for the amino acid sequence of SEQ ID No 2. In a further embodiment, the present invention provides a cell transfected with the nucleic acid sequence of SEQ ID No 1.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will now be described by way of example only, with reference to the accompanying drawings in which:

Figure 1 is the nucleotide (SEQ ID No 1) and deduced amino acid (SEQ ID No 2) sequence of the PERK1 cDNA.

Figure 1A: The DNA sequence corresponding to the PERK1 transcript is shown, with the predicted amino acid sequence presented as a single-letter code below the nucleotide sequence.

Numbers to the left refer to nucleotide sequence and the 5' and 3' untranslated regions are presented in lower case letters. Potential N-glycosylation sites (Asn-x-Ser/Thre) are indicated by dots above the Asn residues, and the extensin signature (Ser-Pro)₄ pentapeptide motif present in the extracellular domain is denoted in boldface type. The predicted membrane spanning region is marked by a solid underline. As defined by Hanks and Quinn (1991), the catalytic domain has been subdivided into 11 subdomains marked by dashed underlines and superscript roman numerals. The amino acids in redface type represent residues that are absolutely conserved, whereas greenface type represent groups of conserved amino acids. The two regions marked by double underlines represent consensus sequences common amongst serine/threonine kinases.

Figure 1B: Structural features of the PERK1 polypeptide. A Kyte hydropathy plot (Kyte and Doolittle, 1982) of the predicted amino acid sequence generated by DNAsis[®] software (Hitachi Software, San Bruno, CA) is shown, where increased hydrophobicity is denoted by positive values. The domains of the PERK1 protein are illustrated below. ECD, extracellular domain; TM, transmembrane domain.

Figure 2 represents the genomic DNA Southern Blot analysis of PERK1.

Genomic DNA (5µg) isolated from *Brassica napus* leaf tissue was digested with the indicated restriction enzymes, blotted and hybridized with a partial 1.5 kb PERK1 cDNA probe under varying conditions of stringency. DNA markers are indicated in kilobases.

Figure 2A: Genomic DNA gel blot analysis under low stringency conditions.

Figure 2B: Genomic DNA gel blot analysis under high stringency conditions.

Figure 3 illustrates the expression of PERK1 cDNA.

Figure 3A: RNA gel blot analysis of PERK1 transcripts in poly(A)⁺ mRNA extracted from various *Brassica napus* tissues. The blot hybridized with a partial 1.5 kb PERK1 cDNA probe detected a full length transcript of ~2.2kb (▷).

Figure 3B: The blot was subsequently probed with cyclophilin as an internal control for even loading (▶).

Figure 3C: Ethidium bromide stain of gel indicates relatively equal amounts of poly(A)⁺ mRNA were loaded.

Figure 4 illustrates a wound-inducible accumulation of PERK1 mRNA in *Brassica napus* leaf and stem tissue.

Figure 4A: Fully expanded leaves were wounded by punching out discs around the perimeter of the leaf blade. Total RNA was extracted at various time intervals after treatment, subjected to Northern blot analysis and probed with full length PERK1 cDNA (▷). The blot was reprobed with cyclophilin used as an internal control for even loading (▶). The graph represents the expression profile of PERK1 in response to wounding corrected against levels of cyclophilin expression. Control unwounded leaf tissue represented by 0 hr time point.

Figure 4B: Northern blot showing a time-course induction of PERK1 mRNA accumulation in wounded stem tissue. Total RNA harvested at the indicated time points was blotted and hybridized against the full length PERK1 coding sequence (▷). The cyclophilin loading control (▶) was used to normalize levels of PERK1 mRNA accumulation represented graphically. Control unwounded stem tissue represented by 0 hr time point.

Figure 5 describes the effects of 50μM methyl jasmonate (MeJA) on PERK1 mRNA accumulation in treated *Brassica napus* leaf and stem tissue.

Figure 5A: *Brassica napus* plants were thoroughly sprayed with a 50μM MeJA solution, and leaf tissue subsequently harvested at different time intervals after treatment. Total RNA prepared from treated leaf tissue was subjected to Northern blot analysis and probed with full length PERK1 cDNA (▷). Control plant (0 hr) was treated with the carrying solution minus the chemical inducer (0.1% [v/v] ethanol for MeJA). The blot was reprobed with cyclophilin used as an internal control for even loading (▶). The graph represents a corrected profile for the levels of PERK1 mRNA accumulation in response to treatment with MeJA normalized against levels of cyclophilin expression.

Figure 5B: Northern blot showing a time-course induction of PERK1 mRNA accumulation in MeJA treated stem tissue. Total RNA harvested at the indicated time points was blotted and

hybridized against the full length PERK1 coding sequence (▷). The cyclophilin loading control (◀) was used to normalize levels of PERK1 mRNA accumulation represented graphically.

Figure 6 illustrates the effects of 4mM salicylic acid (SA) on PERK1 mRNA accumulation in treated *Brassica napus* leaf and stem tissue.

Figure 6A: *Brassica napus* plants were thoroughly sprayed with a 4mM SA solution, and leaf tissue subsequently harvested at different time intervals after treatment. Total RNA prepared from treated leaf tissue was subjected to Northern blot analysis and probed with full length PERK1 cDNA (▷). Control plant (0 hr) was treated with the carrying solution minus the chemical inducer (5mM phosphate buffer, pH7). The blot was reprobed with cyclophilin used as an internal control for even loading (◀). The graph represents a corrected profile for the levels of PERK1 mRNA accumulation in response to treatment with SA normalized against levels of cyclophilin expression.

Figure 6B: Northern blot showing a time-course induction of PERK1 mRNA accumulation in SA treated stem tissue. Total RNA harvested at the indicated time points was blotted and hybridized against the full length PERK1 coding sequence (▷). The cyclophilin loading control (◀) was used to normalize levels of PERK1 mRNA accumulation represented graphically.

Figure 7 is the proposed pathway mediating PERK1 expression in response to wounding, MeJA and SA treatments.

Figure 8 is a Western blot illustrating the bacterially expressed extracellular domain of PERK1.

Figure 8A: Western blot performed on the bacterially expressed extracellular domain fusion protein using a T7 monoclonal antibody which recognizes the T7 epitope on the bacterially expressed fusion protein. There was a marked induction of the fusion protein in the presence of IPTG (Isopropyl-β-d-Thiogalactopyranoside) (Figure 8A; lane 2), however recovery of the purified protein (Lane 4 ; ♦) was not very efficient. The presence of the fusion protein in the insoluble fraction (Lane 3) suggests that the extracellular domain is likely insoluble and could be purified from inclusion bodies.

Figure 8B: Western blot performed to confirm that the extracellular domain fusion protein is targeted to inclusion bodies. Induction of the fusion protein was efficient (Figure 8B; lane2), and despite its presence in the supernatant (Lane 3) and in the denatured insoluble samples (Lane 5), the fusion protein is predominantly in the insoluble fraction (IB-inclusion body) (Lane 4 ; ◊) Lanes 6-9 represent the pTric HisC negative control indicating that the signals obtained for the expression of the extracellular domain protein are specific to the fusion protein.

Figure 9 is a Western blot illustrating the bacterially expressed full length PERK1.

Western blot conducted on the bacterially expressed PERK1 protein to determine whether the full length protein is insoluble and forms inclusion bodies. Although the induction of the full length protein was not as pronounced (Figure 9; lane 2) it is quite evident that the fusion protein is predominantly localized in the insoluble fraction (ie. inclusion body) (Lane 4; ■) Lanes 6-9 represent the parental plasmid as mentioned above.

Figure 10 is a Western blot illustrating the bacterial expression and kinase assay of wild-type and mutated catalytic domains of PERK1.

Figure 10A: A Western blot was performed to confirm the induction and purification of both the wild-type (★) and mutated (◀) catalytic domain fusion proteins using an anti-MBP antibody. The wild-type fusion protein appears to be toxic in bacteria which compromises its inducibility and purification (Figure 10A; lanes 3-4). In addition, the wild-type fusion protein migrates at a slightly larger molecular weight than does the mutated protein. The mutated fusion protein is induced and purified more efficiently, perhaps due to the fact that it is no longer kinase active (Figure 10A ; Lanes 6-7). Purified pMAL-c (Lane 1; ●) was used as a negative control for this experiment.

Figure 10B: A kinase assay performed on affinity purified wild-type and mutated fusion proteins incubated in the presence of γ -³²PdATP. Detection of a phosphoprotein only in Lane 1 provides direct biochemical evidence that the wild-type catalytic domain (★) of PERK1 encodes a functional protein kinase that is capable of autophosphorylation (Lane 1) and that the mutation successfully abolished kinase activity (Lane 2).

Figure 11 illustrates a homology of PERK1 to predicted proteins from the *Arabidopsis* genome sequencing project.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For convenience in the following description, like numerals refer to like structures in the drawings.

The present invention is directed to an isolated gene, PERK1 (Proline Extensin-like Receptor Kinase) (SEQ ID No 1, Figure 1A), which was isolated from λ -pistil cDNA library of *Brassica napus* and represents a novel class of receptor kinases in higher plants. Genomic Southern blot analysis under low and high stringency conditions revealed that PERK1 of the present invention is a single copy gene in the *Brassica* genome (Figures 2A and 2B). PERK1 of the present invention is ubiquitously expressed at high levels in root, stem, petal and pistil and less abundant in leaf tissue (Figure 3A).

In its second aspect, the present invention is directed to PERK1 which, based on its deduced amino acid sequence (SEQ ID No 2, Figure 1A), is predicted to encode a receptor-like kinase possessing an extracellular domain, a single membrane spanning domain and an intracellular kinase domain. The extracellular domain of the protein of the present invention consists of 137 amino acids (Figure 1A) rich in proline and sharing sequence similarity with extensins, a family of hydroxyproline-rich glycoproteins (HRGPs) that constitute a major protein component of higher plant cell walls (Showalter, 1993).

The present invention is further directed to the rapid accumulation of PERK1 mRNA in leaf and stem tissue of *B. napus* in response to wounding (Figure 4) and pathogen attack mimicked by salicylic acid as a chemical elicitor (Figure 6). Therefore, it was suggested that PERK1 of the present invention is a signaling molecule associated with the cell wall via its extensin-like extracellular domain and involved in the transduction of extracellular stimuli (eg. wounding, pathogen attack) into an intracellular response through a cytoplasmic kinase domain, thereby bridging the cell wall - plasma membrane continuum.

In its third aspect, the present invention is directed to the generation of a PERK1 antibody. The coding sequence for the region containing the extracellular domain of PERK1 of the present invention (Figure 8) as well as the coding sequence representing the entire protein

(Figure 9) were cloned into the pTricHis expression plasmid for production of His-tagged fusion proteins in *E. coli* and purification by affinity chromatography on a Talon resin column. The bacterially expressed catalytic domain fusion protein of PERK1 was further tested for kinase activity. The Western blot (Figure 10) confirmed that PERK1 of the present invention encodes a protein with kinase activity.

MATERIALS AND METHODS

Construction of λ -Pistil cDNA Library

Pistils were collected from floral buds of Westar and W1 cultivars 1-2 days before anthesis. Total RNA was isolated using the method described by Jones et al. (1985), and enriched for poly(A)⁺ mRNA by affinity chromatography using pre-packed oligo (dT)₂₅-cellulose beads (New England Biolabs, Beverly, MA). Approximately five micrograms of pistil poly(A)⁺ mRNA was used for the construction of a cDNA library using the ZAP-cDNA[®] synthesis kit (Stratagene, La Jolla, CA). The information encoded by the poly(A)⁺ mRNA was reversed transcribed using MMLV-RT and converted into stable, unidirectional cDNA which was subsequently inserted into a self-replicating Uni-ZAP XR vector, packaged into phage particles in three separate packaging reactions and amplified as described by the manufacturer's procedures (Stratagene, La Jolla, CA). Infection of *Escherichia coli* host strain XLI-Blue yielded a primary library with an average titer of 1.0×10^6 plaque forming units. The primary library was subsequently amplified to obtain an average total of 6.6×10^{10} plaque forming units.

Generation of Putative Novel Receptor-like Protein Kinase Clones

The isolation of putative novel *Brassica napus* receptor kinases relied upon the newly constructed λ -pistil cDNA library and involved *in vivo* mass excision of the pBluecsript phagemids from the Uni-ZAP XR vectors as outlined by the manufacturer (Stratagene, La Jolla, CA). Following efficient mass excision, phagemid DNA was extracted using a large scale alkaline protocol as described by Sambrook et al. (1989) and subjected to the polymerase chain reaction (PCR) using two separate oligonucleotide combinations, RK1/RK2 and RK1/RK3 (obtained from M. Cock, École Normale Supérieure de Lyon, France) specifically designed to prime conserved subdomains of the catalytic domain of receptor protein kinases. RK1 (5'-ggiggTTTCggiAT^TcAgTtTT^AT^TcAA^Aggg-3') served as the forward primer and was constructed based

upon a conserved amino acid consensus (GGFGIV^F/YKG) within subdomain I of the catalytic domain. In the above sequence, and other sequences in this application, the subscripts and superscripts signify variable nucleotide or amino acid residues. The degeneracy of one reverse primer RK2 (5' - AAiATiC^T_giGCCATiCC^A_gAA^A_gT_C - 3') reflects a conserved amino acid consensus (DFGMARIF) of subdomain VII which closely resembles the SRKs in Brassica. The second reverse oligonucleotide RK3 (5' - A^g_AiA^g_AT_CTTigCiA^A_giCC^A_gAA^A_gTC - 3') was generated based upon conserved amino acids (DFGLAKLL) within subdomain VII prevalent among the RLKs isolated in Arabidopsis. Phagemid DNA was amplified in a reaction mixture containing 1µl of excised phagemid DNA, 10x PCR buffer (100mM Tris-HCl pH8.3, 500mM KCl, 15mM MgCl₂), 10mM deoxyribonucleotide triphosphate mixture, 10µM of each oligonucleotide primer and 0.5µl Tsg polymerase (BioBasics, Canada). The PCR reaction was heated at 95°C for 2 min and amplified for 35 cycles under the following amplification conditions: 1 min at 95°C for denaturation, 1 min 30 sec at 50°C for primer annealing and 1 min at 72°C for synthesis. A final extension cycle of 10 min at 72°C was also incorporated into the amplification program. All PCR products generated of the expected size (420-450 bp) were gel purified, cloned into the pT7Blue plasmid (Novagen, Madison, WI) and introduced into *Escherichia coli* DH5α. Transformants were tested for the presence of an insert and positive clones were sequenced with universal primers (R-20 and U-19) by an ABI automated sequencer (Model 373 STRETCH DNA; Perkin Elmer Corp., Canada Ltd.) using the dideoxychain-terminating method described by Sanger et al. (1977). Sequence analyses performed using DNAsis[®] software (Hitachi Software, San Bruno, CA) at the nucleotide and amino acid levels yielding homologies less than 85% and 70% respectively were considered novel and led to the isolation of four putative novel *B.napus* kinases, one of which is PERK1.

Screening of λ-Pistil cDNA Library

The original 351 bp PCR product was used to screen the λ-pistil cDNA library. Approximately 2x10⁶ plaques from the amplified library were screened and plated at a density of 1x10⁵ pfu/plate. Duplicate colony lifts were performed according to Sambrook et al. (1989), and prehybridized for 2 hr at 42°C in 50% formamide, 5x Denhardt's solution (1x Denhardt's solution is 0.02% Ficoll, 0.02% DVP, 0.02% BSA), 5x SSC (1x SSC is 0.15M NaCl, 0.015M sodium citrate), 0.1% SDS, 1mM EDTA and 100µg/ml salmon sperm DNA. Filters were

subsequently hybridized overnight in the same solution containing the 351 bp PERK1 cDNA radiolabeled by random priming (Feinberg and Vogelstein, 1983) and washed twice with 2x SSC, 0.1% SDS at room temperature for 15 min, followed by two 25 min washes with 0.5x SSC, 0.1% SDS at 55°C. Plaques containing putative positive clones were cored and subjected to several rounds of screening until single isolates representing the PERK1 clone were obtained. Single clone excision to liberate the double stranded pBluescript phagemid was performed on each isolate according to the procedure recommended by the manufacturer (Stratagene, La Jolla, CA). Phagemid DNA digested with EcoRI/XhoI to release the cloned cDNA was subjected to standard plasmid Southern blot analysis as described by Sambrook et al. (1989) and probed with the radiolabeled 351 bp PERK1 cDNA. The membrane was prehybridized at 42°C in 5x SSPE, 10x Denhardt's solution and 0.5% SDS for 2 hr and hybridized overnight at the same temperature in a buffer containing 50% formamide, 5x SSPE and 0.5% SDS. Washing conditions were performed twice at room temperature for 15 min in 2x SSC, 0.1% SDS followed by several 30 min washes at 55-60°C in 0.1x SSC, 0.1% SDS. An intense hybridization signal would confirm whether phagemids isolated from the library screen contained the cloned cDNA of interest. Several positive clones were sequenced as previously mentioned using both universal and sequence specific primers to generate a consensus sequence representing the PERK1 cDNA clone (1512bp) isolated from the λ -pistil cDNA library.

Rapid Amplification of cDNA Ends (5'RACE)

The 5' end of the PERK1 cDNA was obtained by the procedure for the rapid amplification of cDNA ends originally described by Frohman et al. (1988) using the 5' RACE System, Version 2.0 kit (Gibco-BRL, Gaithersburg, MD). First strand cDNA was synthesized from approximately 300µg of mixed Westar and W1 pistil total RNA using a gene specific primer GSP1 (5'-TAACCAACAAGAgACA-3') designed to anneal approximately 300 bp from the 5' end of the PERK1 cDNA (1512 bp) isolated from the library screen. Following cDNA synthesis, the first strand product was purified from unincorporated dNTPs and GSP1 using a GLASS MAX[®] spin cartridge. A homopolymeric tail was added to the 3' end of the cDNA using TdT (terminal deoxynucleotidyl transferase) and dCTP. Tailed cDNA was amplified using a second gene specific primer GSP2 (5'-CCACTCCCAACTTTCAAC-3') designed to anneal 3' to GSP1 with respect to the cDNA, and an abridged anchor primer (Gibco-BRL, Gaithersburg, MD) which

annealed to the homopolymeric tail. PCR amplification was carried out for 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 2 min, followed by a final extension cycle for 10 min. A PCR product of the expected size (~1 kb) corresponding to the 5' end of PERK1 was gel purified, cloned into the pT7Blue plasmid (Novagen, Madison, WI) and transformed into *Escherichia coli* DH5α. Confirmation of the 5'RACE product was obtained by plasmid Southern blot analysis as described above and by sequential primer based sequencing.

Cloning of Full Length PERK1 cDNA

A PCR based approach was used to generate a full length PERK1 cDNA by combining the 5'RACE product cloned into the EcoRV site of pT7Blue with the cDNA isolated from the library screen cloned into the EcoRI/XhoI sites of the pBluescript SK phagemid. A forward primer (5'-ggAAAgCTTgCATgCCTgCAGgTCgAC-3') containing an internal PstI site was designed to anneal upstream to the EcoRV cloning site of pT7Blue. A reverse primer (5'-CgCCTgCAGgTAATACgACTCACTATAggg-3') also containing a PstI site was designed based on pBluescript phagemid sequence immediately 3' to the EcoRI/XhoI cloning site. Full length PERK1 cDNA was generated from a 100μl PCR reaction containing 1μl (~20ng) of each template (cDNA in pT7Blue and pBluescript phagemid), 10x Pfu Buffer (200mM Tris-HCl pH8.8, 100mM (NH₄)₂SO₄, 20mM MgSO₄, 1% Triton®X-100, 1mg/mlBSA), 10mM dNTPs, 50pmol forward and reverse primers and 1μl Pfu polymerase (Gibco-BRL, Gaithersburg, MD). The samples were heated to 94°C for 5 min and amplified for 30 cycles with a denaturing cycle of 1 min, a primer annealing cycle at 53°C for 1 min followed by an extension cycle for 3 min at 72°C. The resulting PCR product of the expected size (~2.2kb) was gel purified and cloned into the PstI restriction site of pBluescript KS (+/-) II. The full length PERK1 cDNA sequence was confirmed by a sequential primer based sequencing approach using both universal and sequence specific primers as previously described. All DNA and protein sequence analysis was performed using the DNAsis® Software (Hitachi Software, San Bruno, CA).

Genomic DNA Isolation and Southern Blot Analysis

Genomic DNA was extracted from approximately one gram of young *Brassica napus* leaf tissue according to the method described by Goring et al. (1992b). Approximately 5μg of

genomic DNA was digested with several restriction enzymes (BamHI, EcoRI, HindIII, PstI, XbaI, XhoI), fractionated through a 0.8% agarose gel and transferred overnight in 10x SSC onto Zetaprobe membrane (Biorad, Hercules, CA). This was performed in duplicate to test hybridization conditions under low and high stringencies conditions. After drying, the membranes were prewashed in 0.1x SSC, 0.5% SDS for 25 min at 60°C. The membranes were prehybridized and hybridized as previously described for plasmid Southern blots with the inclusion of 10% dextran sulfate and 50µg/ml salmon sperm DNA in the hybridization buffer. Washing conditions for genomic southern blots varied depending on the stringency tested. One membrane was washed under conditions of low stringency for 15 min at room temperature in 2x SSC, 0.1% SDS followed by second 15 min room temperature wash in 1x SSC, 0.1% SDS and three final washes at 50°C in 1x SSC, 0.1% SDS.. The second membrane was washed under conditions of high stringency by lowering the salt concentration to 0.1x SSC, 0.1% SDS and increasing the temperature to 65°C. The ³²P-labeled 1512 bp PERK1 cDNA probe was generated by random priming as described by Feinberg and Vogelstein (1983). Membranes were subjected to autoradiography (XAR-5 film, Kodak) overnight at -80°C.

Isolation and Northern Blot Analysis of Multiple Tissue RNA

Total RNA was extracted from a mixture of Westar and W1 root, stem, leaf, petal, anther and pistil tissue as described by Jones et al. (1985). Poly(A)⁺ mRNA was isolated using the polyA SpinTM mRNA Isolation kit as outlined by the manufacturer's procedure (New England Biolabs, Beverly, MA). Approximately 3µg of poly(A)⁺ mRNA was fractionated on a 1.2% formaldehyde gel (Sambrook et al., 1989) and transferred to Zetaprobe membrane (Biorad, Hercules, CA) in 10x SSC. Hybridization and high stringency wash conditions were conducted as previously described for genomic Southern blot analysis. The membrane was subsequently probed with a cyclophilin EST clone (No. mBN086) as an internal control for even loading of poly(A)⁺ mRNA.

Plant Treatments

B. napus plants were grown in a growth chamber at 22°C with a 16hr light period followed by an 8hr dark period at 16°C. Experiments were conducted on two month old plants, and all experiments used one plant per time point from which leaf and stem tissue was harvested.

Wounding of leaf material was performed by punching out leaf discs every 1cm around the perimeter of the leaf blade ensuring that the midvein remained intact, and stems were wounded by slicing into 1-3cm segments. The wounded tissues were placed in petri dishes containing filter paper moistened with 20mM sodium phosphate buffer supplemented with 50µg/ml chloramphenicol to prevent bacterial contamination of the wounded tissue (Shirsat et al., 1996). A control (0 hr) time point for this experiment was performed by incubating unwounded tissue in the sodium phosphate buffer. Wounded leaf and stem tissue was harvested at different times after wounding (0 hr, 5 min, 15 min, 45 min, 1 hr, 4 hr, 12 hr, 24 hr, 36 hr, 48 hr).

Plants were thoroughly sprayed with 50µM methyl jasmonate (MeJA; Sigma, St. Louis, MO) (Titarenko et al., 1997) and 4mM salicylic acid (SA; Sigma, St. Louis, MO) solutions (Schweizer et al., 1998). Leaf and stem tissue was harvested at various time points (0 hr, 5 min, 15 min, 30 min, 45 min, 1 hr, 2 hr, 4 hr, 12 hr, 24 hr, 36 hr, 48 hr, 72 hr, 96 hr) following SA and MeJA treatments. Control sprays were performed with the carrying solutions in the absence of the chemical inducer. Carrying solutions were 5mM phosphate buffer, pH 7 for SA, and 0.1% [v/v] ethanol for MeJA.

Total RNA was extracted from treated tissue according to the method described by Cock et al. (1997). Depending on the treatment, varying amounts of total RNA (20-40µg) was electrophoresed on a 1.2% formaldehyde gel and standard Northern blot analysis was performed as described by Sambrook et al. (1989). Hybridization and washing conditions were performed as outlined for the multiple tissue northern blot. Following autoradiography, the amounts of radioactive signal were quantified using Instant Imager Electronic Autoradiography (Packard, Meriden, CT). The membranes were reprobed with the cyclophilin cDNA and the amounts of hybridized radiolabeled cyclophilin were quantified in the same manner. The relative amounts of RNA hybridized to the full length PERK1 cDNA probe were determined after correction for differences in the amounts of cyclophilin RNA.

RESULTS

Isolation and Sequence Analyses of PERK1 cDNA

In order to isolate novel receptor-like protein kinases in *B. napus* a combination of degenerate oligonucleotide primers designed against conserved kinase subdomains I and VII (Hanks and Quinn, 1991) were used to amplify mass excised phagemid DNA from a newly constructed λ -pistil cDNA library. The cDNAs encoding products of the expected length (~420-450 bp) were cloned and the deduced partial sequences were analysed against several databases in order to determine which clone represented a putative novel kinase. One of several candidates, showed the highest degree of sequence similarity to the cytoplasmic domain of known plant receptor protein kinases, and was therefore used to screen the amplified λ -pistil cDNA library. Several positive clones obtained from the library screen were completely sequenced and a partial 1512 bp consensus sequence was generated to represent the PERK1 cDNA isolated from the library screen. Although this partial PERK1 cDNA had an open reading frame, it did not encode a full length transcript, therefore the 5' end was completed by 5' RACE (see Methods).

The deduced amino acid sequence of PERK1 is shown in Figure 1A and a schematic representation of its hydropathy plot is shown in Figure 1B. The full length cDNA sequence is 2189 bp and consists of one large open reading frame of 1944 bp encoding a predicted protein of 648 amino acids with an estimated molecular mass of 69 kDa (Figure 1). The first methionine of this open reading frame is preceded by two in frame stop codons, TAA and TGA at positions -48 to -45 and -23 to -21 respectively. In addition, there is also an AGAA sequence at position -9 to -6 (Figure 1) which is a favourable site for translation initiation in all eukaryotes (Lutcke et al., 1987).

Based on this sequence, PERK1 is predicted to encode a receptor-like kinase possessing an extracellular domain, a single membrane spanning domain and an intracellular kinase domain (Figure 1B) with three potential N-linked glycosylation sites (Asn-X-Ser/Thr) found throughout the sequence (Figure 1A) (Weinstein et al., 1982). The predicted polypeptide sequence was analyzed using the PSORT database and determined to be a Type Ib intergral membrane protein with a hydrophilic amino terminal domain exposed on the exterior of the membrane but whose coding sequence does not indicate a cleavable signal sequence preceding this domain. Singer

(1990) proposes that despite the lack of a signal peptide, Type Ib integral membrane proteins are inserted into the membrane via the usual ER-translocator protein machinery with some slight modifications. The extracellular domain of this protein consists of 137 amino acids (Figure 1A) rich in proline and sharing sequence similarity with extensins, a family of hydroxyproline-rich glycoproteins (HRGPs) that constitute a major protein component of higher plant cell walls (Showalter, 1993). Extensin proteins have two proposed functions in plants, one which contributes to the structural support of the cell wall by forming glycoprotein networks and the other which involves plant defense; helping to protect the plant against mechanical wounding or pathogen attack (Wilson and Fry, 1986; Showalter, 1993). A distinctive characteristic prevalent among dicot extensins is the repetitive Ser-(Pro)₄ pentapeptide consensus motif (Showalter, 1993). A unique feature of PERK1's extracellular domain is the predominance of a slightly modified Ser-(Pro)₂₋₃ motif in addition to the presence of one signature pentapeptide block (Figure 1A). In order to investigate the phylogenetic status of PERK1, sixty four deduced amino sequences corresponding to the extracellular and transmembrane domains of extensin, proline rich and other cell wall proteins were retrieved from Genbank and used to construct a phylogenetic tree (Clustal X). Results from the phylogenetic analysis indicated that PERK1 is most similar to a subset of extensin proteins as suggested by the sequence homology restricted predominantly to the serine/proline rich regions of the protein (data not shown).

The predicted protein also contains two other domains of note. Hydropathy analysis (Kyte and Doolittle, 1982) of the protein sequence predicted a membrane spanning region of 23 amino acids (Figure 1; residues 138-160) followed by a characteristic stop transfer sequence rich in charged amino acids [Arg-Arg-Arg] required for the proper insertion in the membrane (Weinstein et al., 1982).

All known protein kinases display amino acid sequence similarity in their catalytic domains which are comprised of eleven subdomains containing some invariant residues important for catalysis (Hanks and Quinn, 1991). The overall features of this organization are identified in the catalytic domain (residues 274-548) of the PERK1 protein in that all of the absolutely conserved amino acids as well as the highly conserved amino acid groups are present (Figure 1A). The sequences of DIKASN in subdomain VI and GTFGYLAPE in subdomain VIII (Hanks and Quinn, 1991) are strong indicators that PERK1 may possess serine/threonine

rather than tyrosine substrate specificity (Figure 1A) which likely suggests a role for PERK1 in plant signal transduction (Hanks and Quinn, 1991)..

PERK1 is a Single Copy Gene and Ubiquitously Expressed in *B. napus* Tissue

As shown in Figure 2, Southern blot analysis was performed under conditions of varying stringency using *B. napus* genomic DNA digested with several restriction enzymes in order to determine copy number of PERK1 in the Brassica genome. Based on known restriction sites within the cDNA and identical hybridization patterns obtained for low and high stringency conditions (Figure 2), PERK1 appears to exist as a single copy gene and is not a member of a multigene family.

In order to determine whether PERK1 is expressed in plant tissues, RNA gel blot analysis was performed using poly(A)⁺ mRNA isolated from a variety of *B. napus* tissues as shown in Figure 3. A partial 1.5 kb PERK1 cDNA probe used in this experiment detected a transcript of 2.2 kb (Figure 3A) which is consistent with the size of the full length PERK1 cDNA. The presence of a higher molecular weight transcript of ~ 4.4 kb in root (Figure 3A) may represent an alternatively spliced transcript of PERK1 generated during mRNA processing. This is reminiscent of the alternative transcript detected for the carrot extensin gene which as reported by Chen and Varner (1985) is predominantly expressed in root tissue.

The 2.2 kb PERK1 transcript was most abundant in *B. napus* root, stem and petal tissue (Figure 3A). Levels of PERK1 mRNA were also detected in leaf and pistil tissue albeit at much lower levels and transcripts appear to be absent in the anther tissue (Figure 3A). As an internal control the blot was reprobed with a cyclophilin EST cDNA to ensure even loading of the poly(A)⁺ mRNA. A 700 bp cyclophilin transcript detected with relatively the same intensity in all tissues with the exception of anther indicates that equal amounts of mRNA was used (Figure 3B). In addition, the ethidium bromide stained gel shows relatively even loading (Figure 3C). The striking difference in the intensity of the cyclophilin signal in anther tissue (Figure 3B) is a common problem associated with the desiccate nature of the tissue, and could be attributed to the low abundance of cyclophilin mRNA present in the anther tissue as reported by Gasser et al. (1990).

Changes in PERK1 mRNA in Response to Wounding and Chemical Elicitors

The increasing evidence that protein kinases play important roles in plant defense (Zhou et al., 1995; Usami et al., 1995; Suzuki and Shinji, 1995), in addition to the significant homology shared between the extracellular domain of PERK1 and extensin proteins prompted us to address a possible role for PERK1 in mediating plant responses to mechanical wounding and pathogen attack. In contrast to extensin genes isolated from many other plant species including *Arabidopsis* (Merkouropoulos et al., 1999), carrot (Chen and Varner, 1985), tobacco (Keller and Lamb, 1989) and tomato (Showalter et al., 1991) which are primarily comprised of tandem repeats of the extensin motif, a striking and unique feature in the organization of PERK1 is the presence of a catalytic domain. It is conceivable that PERK1 is a signaling molecule associated with the cell wall via its extensin-like extracellular domain and involved in the transduction of extracellular stimuli (eg. wounding, pathogen attack) into an intracellular response through a cytoplasmic kinase domain, thereby bridging the cell wall - plasma membrane continuum.

In order to examine whether PERK1 expression could be influenced by external stimuli, leaf and stem tissue of *B. napus* plants were wounded and the abundance of PERK1 mRNA was determined by standard Northern blot analysis using the full length PERK1 cDNA as a probe (see Methods). Figure 4 shows changes in the steady-state levels of PERK1 mRNA accumulation following injury. PERK1 transcripts in wounded leaf tissue began to accumulate 5 min after wounding, reaching maximal levels within 15 min post injury represented by an 8.2 fold induction. A 4.5 fold increase in PERK1 mRNA levels was detected 45 min following treatment declining towards basal levels by 1 hr (Figure 4A).

A similar profile of PERK1 mRNA steady state levels was obtained for wounded stem tissue (Figure 4B). An accumulation of PERK1 mRNA in stem is evident 15 min following wounding which represents a 3.6 fold induction of this gene. Maximum steady state levels of PERK1 mRNA in stem was achieved 45 min after injury corresponding to a 4.5 fold induction. Therefore, the overall kinetics of PERK1 mRNA accumulation in both tissues after mechanical wounding is clearly a very rapid response (Figure 4).

Defense mechanisms deployed by plants in response to wounding or pathogen attack have been shown to be induced by certain plant derived chemicals such as methyl jasmonate (MeJA) and salicylic acid (SA). In order to examine changes in the levels of PERK1 mRNA abundance in response to exogenous application of MeJA, *B. napus* plants were thoroughly sprayed with a 50µM MeJA solution. Leaf and stem tissue was subsequently harvested at various times and the steady state levels of PERK1 mRNA were analysed. Figure 5A shows the RNA gel blot and corrected PERK1 mRNA profile for treated leaf tissue during which no significant accumulation of PERK1 mRNA was detected. In response to MeJA, levels of PERK1 transcript in leaf tissue were very weak resembling basal levels in untreated tissue (Figure 3A). Exogenous application of MeJA to stem tissue had no effect on the accumulation of PERK1 mRNA as shown by the corrected profile in which the fold induction of PERK1 did not deviate substantially from the untreated control (0 hr) (Figure 5B). Furthermore, no increase in the steady state levels of PERK1 mRNA was detected in the appropriate control treatment (0.1% [v/v] ethanol, solvent control for MeJA) at time 0 hr.

Despite the well established involvement of MeJA (the methyl ester of the plant growth regulator jasmonic acid (JA)) in the signal transduction pathway regulating gene activation upon wounding, processes occurring immediately after wounding remain poorly characterized in terms of additional components that may also participate in wound signaling (Titarenko et al., 1997). Steady state levels of PERK1 mRNA remain unaffected to exogenously applied MeJA (Figure 5) which suggests that the inducibility of PERK1 by wounding occurs via a MeJA-independent pathway (Figure 7). Studies conducted by Titarenko et al. (1997) addressing the role of JA in mediating wound responses support the existence of multiple distinct wound signal transduction pathways. Exogenously applied JA was able to induce only a subset of wound responsive genes in Arabidopsis which ultimately resulted in a stronger systemic accumulation in wounded plants. Conversely, a second set of wound responsive genes showing a stronger induction locally in wounded tissue showed no substantial accumulation upon JA treatment. In conjunction with the pattern of PERK1 mRNA accumulation in response to wounding and MeJA, it appears that plants respond to wounding by two distinct wound signal transduction pathways: one which does not require JA and is primarily responsible for gene activation in the vicinity of the wound site and the other which involves JA perception and most likely activates gene expression both locally and systemically to the wound site (Titarenko et al., 1997).

Many genes isolated to date that are induced by a pathogenic stimulus can be at least partially induced by SA (Ward et al., 1991). In order to address the potential role of PERK1 in a plant's defense response against pathogen attack, 4mM SA was used as a chemical elicitor and sprayed onto *B. napus* plants. Figure 6A shows that when SA is exogenously applied to leaf tissue, PERK1 mRNA accumulates 15 min following treatment reaching a maximum 5 fold induction 45 min post-treatment. Steady state levels of PERK1 mRNA in treated stem tissue peaked at 45 min corresponding to an approximate 2 fold induction in response to 4mM SA (Figure 6B).

Collectively, the profiles of PERK1 mRNA accumulation in response to wounding, MeJA and SA are not entirely surprising. PERK1 induction is rapid in response to wounding (Figure 4) and the lack of PERK1 transcript accumulation in response to MeJA (Figure 5) suggests a pathway for wound mediated induction of PERK1 that is independent of MeJA (Figure 7). The pronounced and rapid induction of PERK1 in response to exogenous SA (Figure 6) supports other studies showing that SA is known to inhibit wound responsive genes that are regulated by a MeJA-dependent pathway (Peña-Cortés et al., 1993; Doares et al., 1995). Therefore, it is unlikely that both MeJA and SA would induce PERK1 mRNA accumulation given that these pathways are known to be antagonistic (Peña-Cortés et al., 1993). Nevertheless, the rapid induction of PERK1 during these treatment suggests a potential role early on in a plant's defense signaling pathway.

Protein work on PERK1

The generation of a PERK1 antibody would be an invaluable tool in studies to address the expression, localization, regulation and potential function of PERK1 of the present invention. The coding sequence for the region containing the extracellular domain of PERK1 of the present invention as well as the coding sequence representing the entire protein were cloned into the pTricHis expression plasmid for production of His-tagged fusion proteins in *E. coli* and

purification by affinity chromatography on a Talon resin column.

Figure 8A represents a Western blot performed on the bacterially expressed extracellular domain fusion protein using a T7 monoclonal antibody which recognizes the T7 epitope on the

bacterially expressed fusion protein. There was a marked induction of the fusion protein in the presence of IPTG (Isopropyl- β -d-Thiogalactopyranoside) (Figure 8A; lane 2), however recovery of the purified protein (Lane 4 ; ♦) was not very efficient. The presence of the fusion protein in the insoluble fraction (Lane 3) suggests that the extracellular domain is likely insoluble and could be purified from inclusion bodies. This method relies on the bacterial expression system generating large amounts of the insoluble fusion protein which form inclusion bodies in the bacterial host. Figure 8B is a Western blot to confirm that the extracellular domain fusion protein is targeted to inclusion bodies. Induction of the fusion protein was efficient (Figure 8B; lane2), and despite its presence in the supernatant (Lane 3) and in the denatured insoluble samples (Lane 5), the fusion protein is predominantly in the insoluble fraction (IB-inclusion body) (Lane 4 ; ◊) Lanes 6-9 represent the pTric HisC negative control indicating that the signals obtained for the expression of the extracellular domain protein are specific to the fusion protein. The extracellular domain fusion protein can be used as an antigen and injected into rabbits in order to generate a polyclonal antibody against the extracellular domain of PERK1. This fusion protein can either be injected directly as an inclusion body in the presence of an adjuvant, or alternatively, the protein purified from the inclusion bodies can be separated on an SDS-Polyacrylamide gel, and the region of the gel at which the protein of interest migrates can be excised and used for injection. It should be noted that the bacterially expressed fusion protein is migrating at a higher molecular weight than predicted from the amino acid sequence, however this is not entirely uncommon and could in fact be due to the high percentage of proline residues (40%) comprising the extracellular domain of PERK1.

Similar experiments were conducted for the fusion protein corresponding to the entire coding region of PERK1. Figure 9 is a Western blot conducted on the bacterially expressed PERK1 protein to determine whether the full length protein is insoluble and forms inclusion bodies. Although the induction of the full length protein was not as pronounced (Figure 9; lane 2) it is quite evident that the fusion protein is predominantly localized in the insoluble fraction (i.e. inclusion body) (Lane 4; ■) Lanes 6-9 represent the parental plasmid as mentioned above. We are currently modifying several variables of the induction protocol in an attempt to maximize the yield of full length PERK1 protein. Once these conditions are optimized, the PERK1 fusion

protein can also be used as an antigen to raise antibody against full length PERK1 as previously described.

In order to determine whether PERK1 encodes a protein with kinase activity as its sequence predicts, the bacterially expressed catalytic domain fusion protein of PERK1 was tested for kinase activity. Furthermore, to ensure that the phosphorylation of the fusion protein was not a result of bacterial kinase activity, a mutated catalytic domain was also generated by site directed mutagenesis which introduced a single base pair substitution of a lysine residue to a glutamic acid residue (L → E). This mutation modifies the essential invariant lysine of subdomain II required for phospho-transfer and renders the kinase inactive. Both the wild-type and the mutated catalytic domains of PERK1 were cloned into the pMAL-c expression system, induced for protein production in the presence of IPTG and purified by affinity chromatography on MBP amylose resin. Figure 10A is a Western blot to confirm the induction and purification of both the wild-type (★) and mutated (◀) catalytic domain fusion proteins using an anti-MBP antibody. The wild-type fusion protein appears to be toxic in bacteria which compromises its inducibility and purification (Figure 10A; lanes 3-4). In addition, the wild-type fusion protein migrates at a slightly larger molecular weight than does the mutated protein. The mutated fusion protein is induced and purified more efficiently, perhaps due to the fact that it is no longer kinase active (Figure 10A ; Lanes 6-7). Purified pMAL-c (Lane 1; ●) was used as a negative control for this experiment.

Figure 10B represents a kinase assay performed on affinity purified wild-type and mutated fusion proteins incubated in the presence of γ -³²PdATP. Detection of a phosphoprotein only in Lane 1 provides direct biochemical evidence that the wild-type catalytic domain (★) of PERK1 encodes a functional protein kinase that is capable of autophosphorylation (Lane 1) and that the mutation successfully abolished kinase activity (Lane 2).

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Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention as outlined in the claims appended hereto.

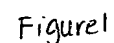
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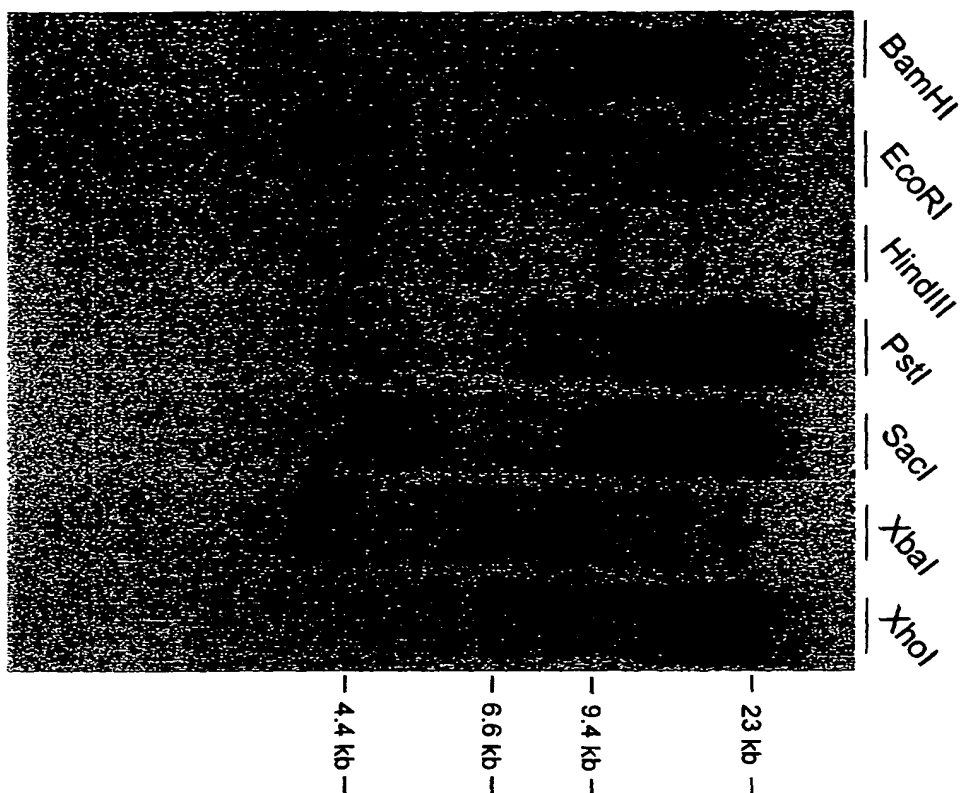
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B.



A.



B.

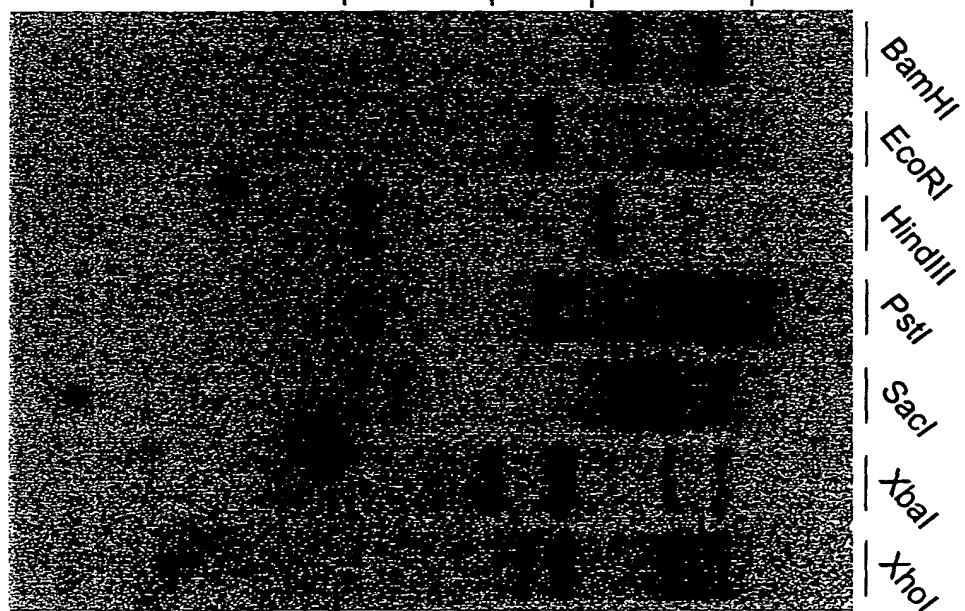


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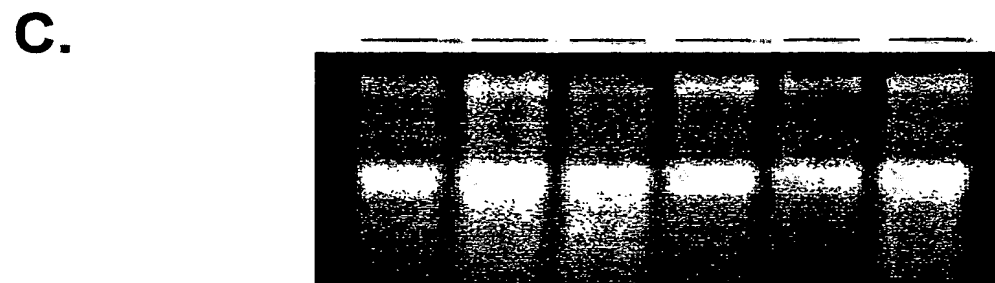
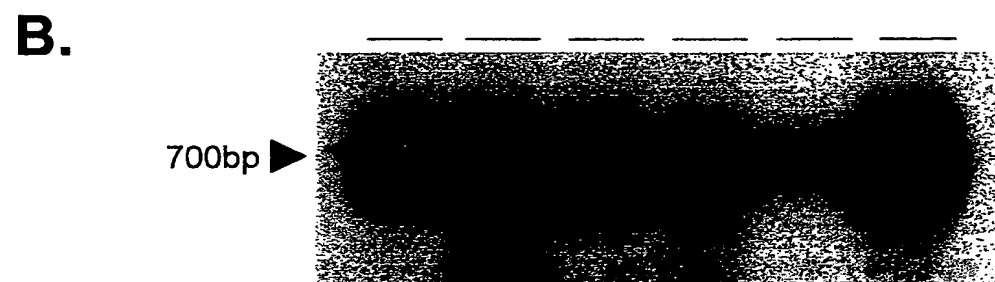
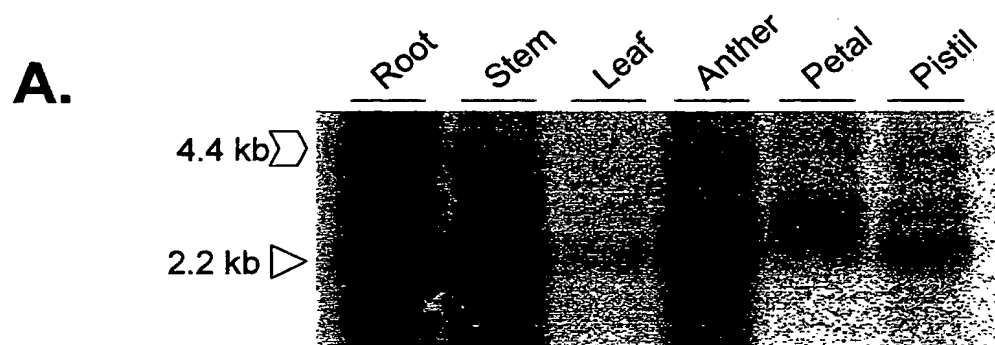
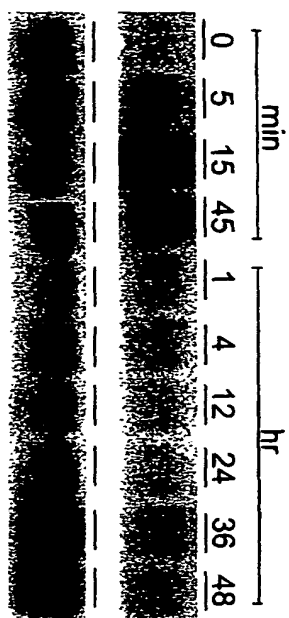


Figure 3

A.



B.

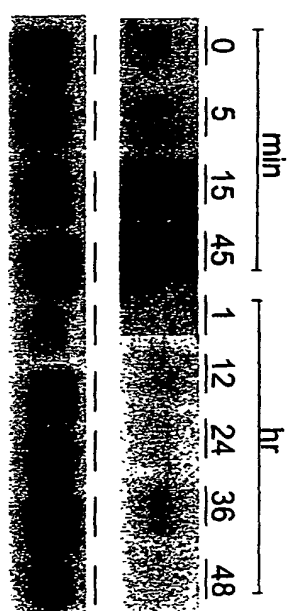
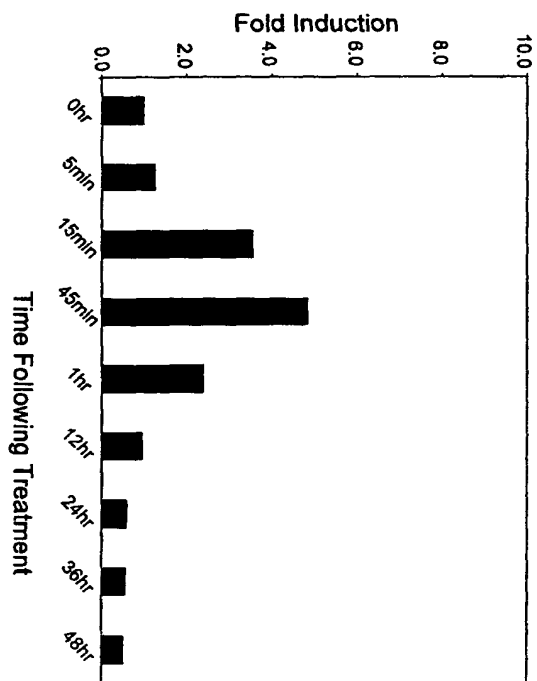
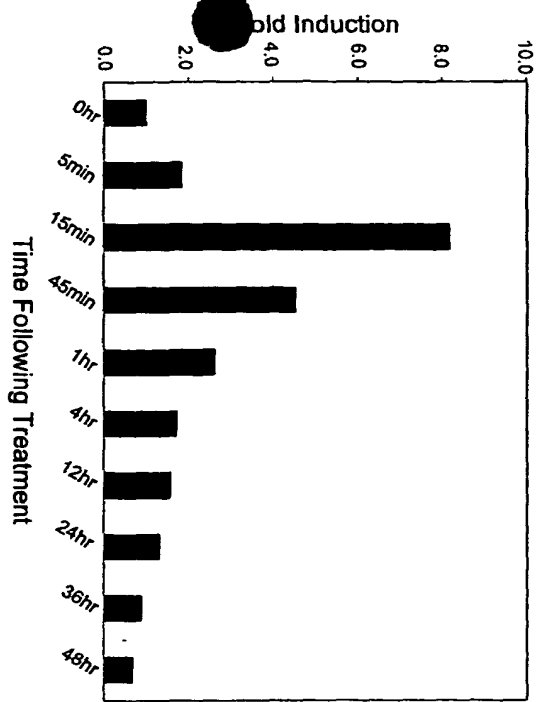
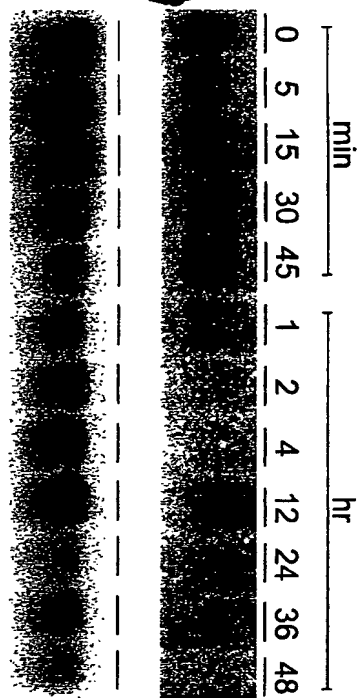


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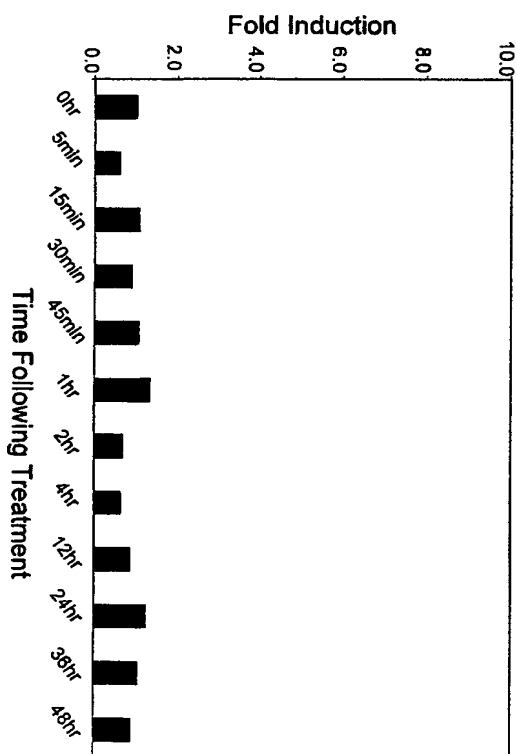
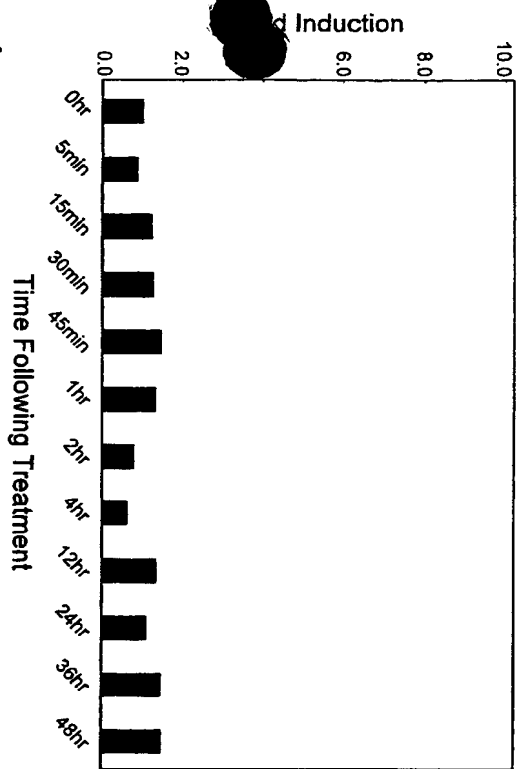
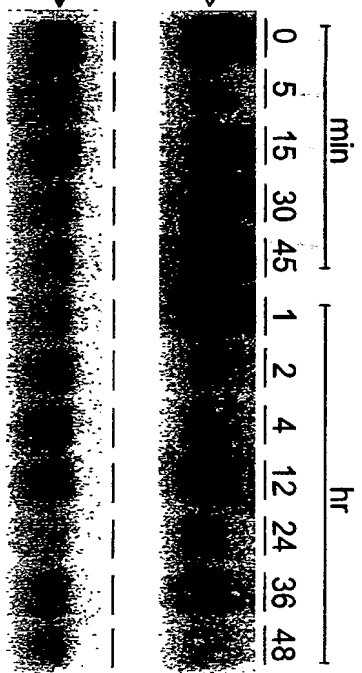


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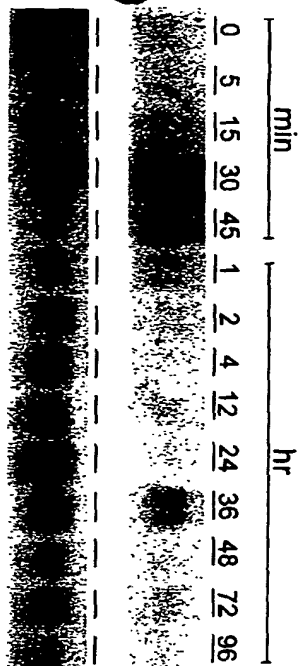


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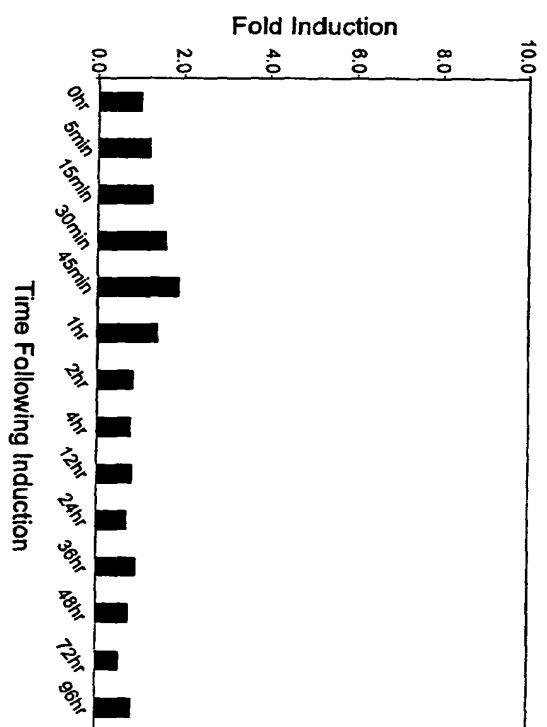
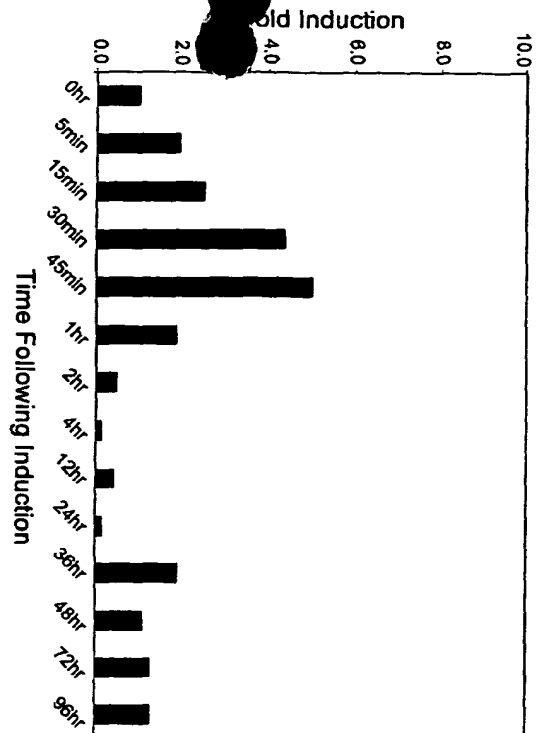
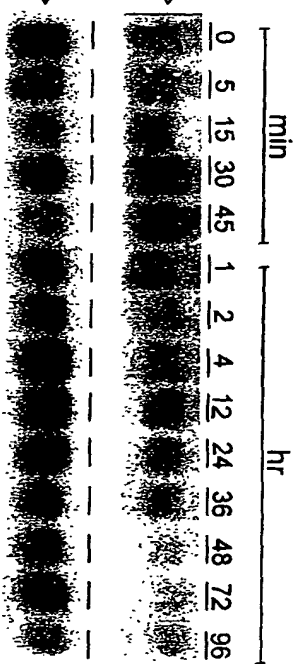


Figure 6

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Mechanical Wounding
or
Insect Attack

Pathogen
Attack
(eg. bacterial, fungal)

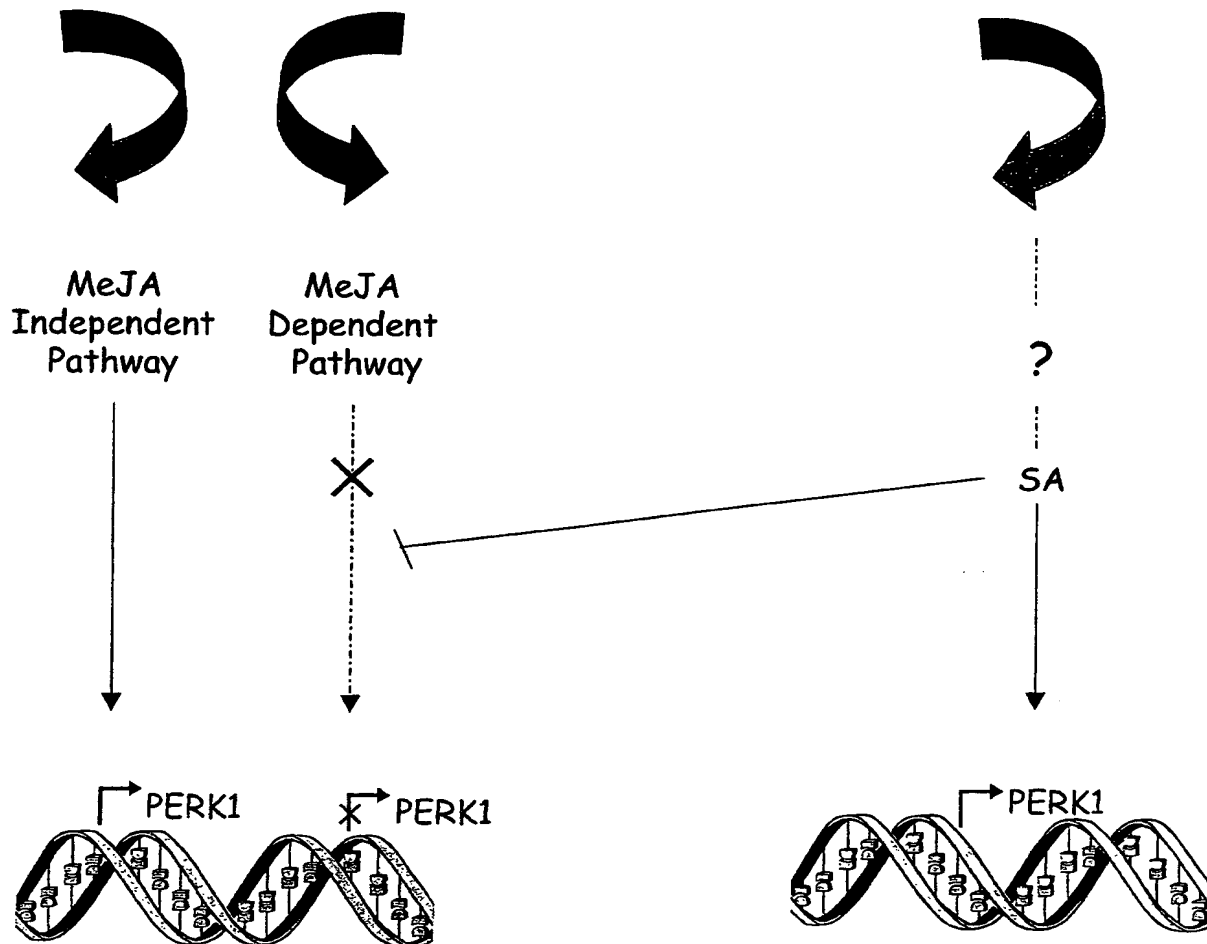


Figure 7

Bacterially Expressed Extracellular Domain of PERK1

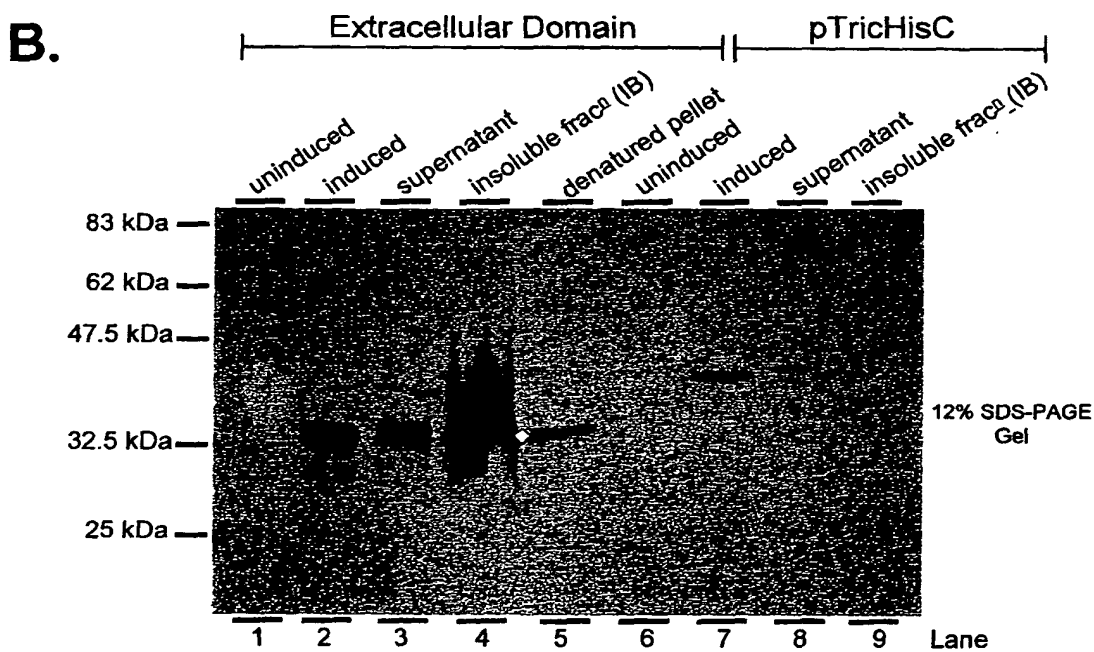
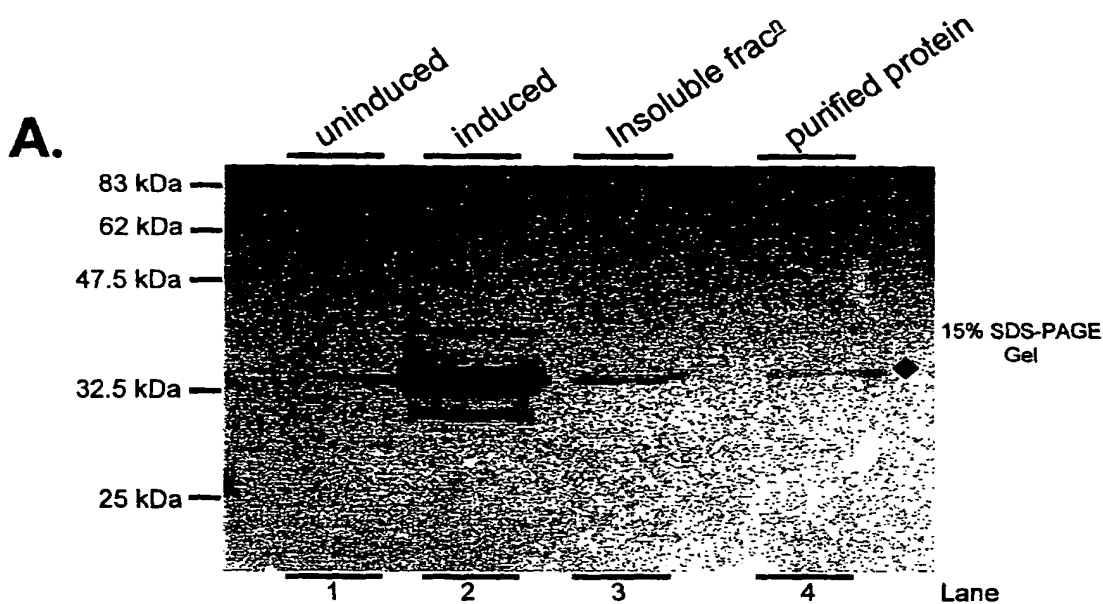


Figure 8

Bacterially Expressed Full Length PERK1

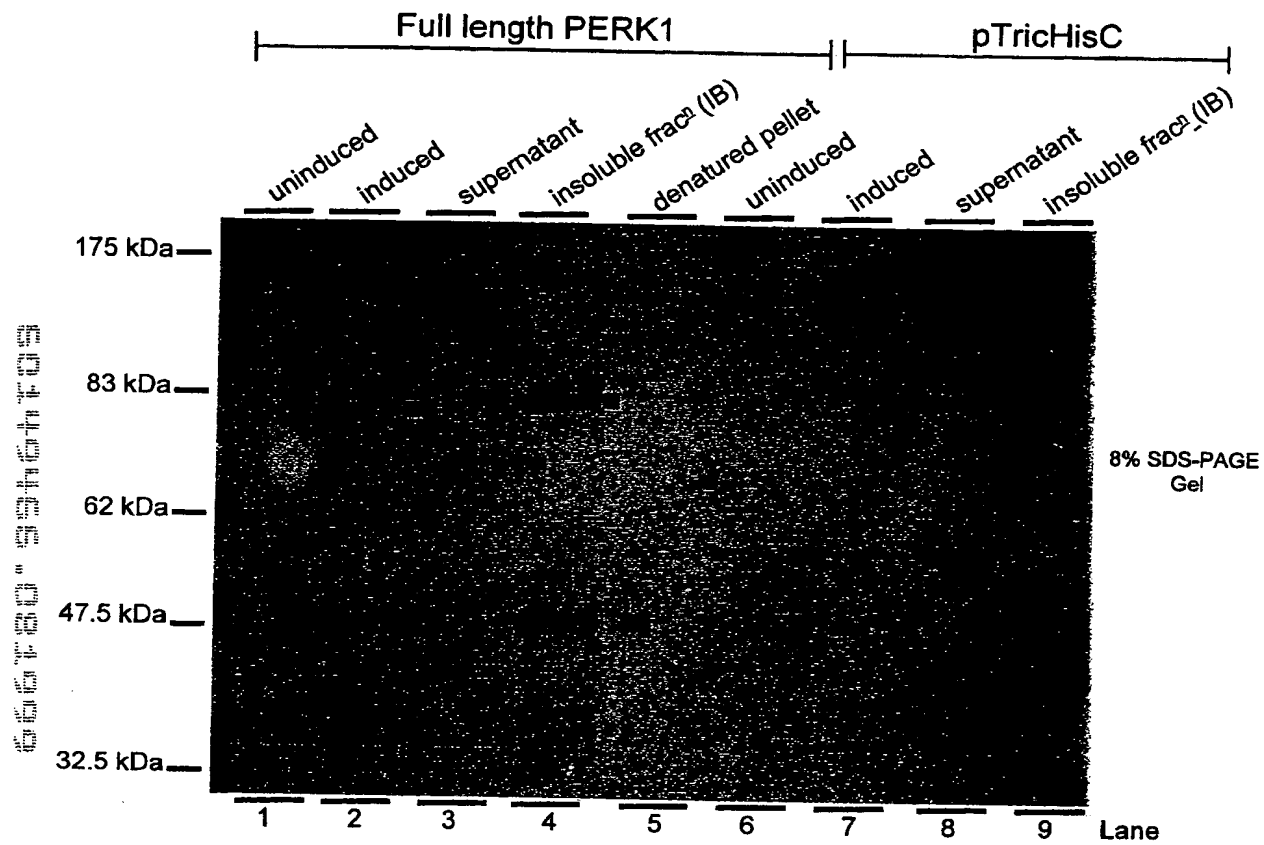
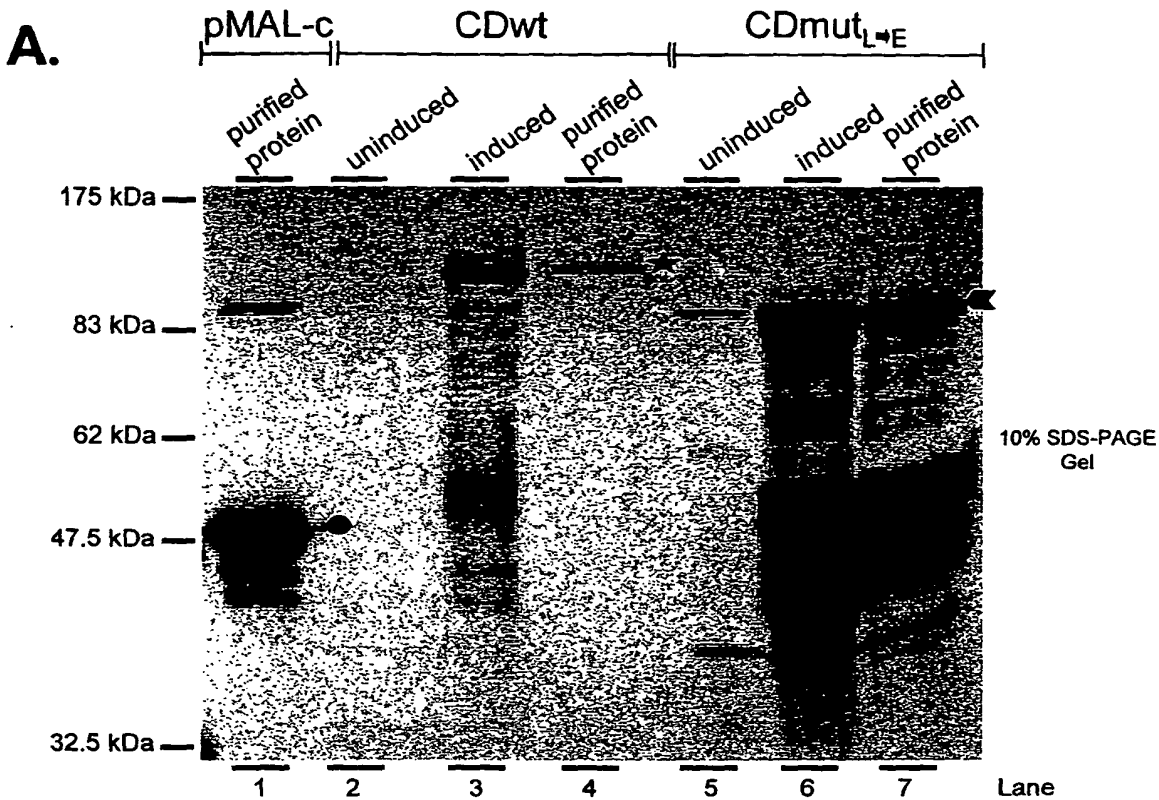


Figure 9

Bacterial Expression and Kinase Assay of Wild-Type and Mutated Catalytic Domains of PERK1



B.

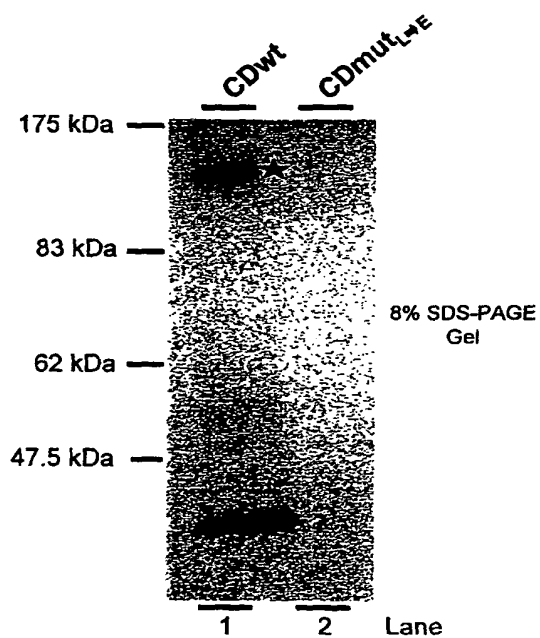
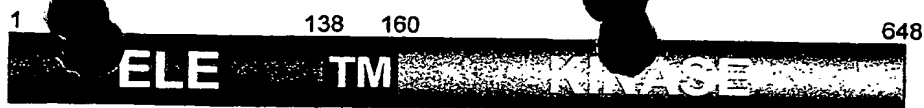
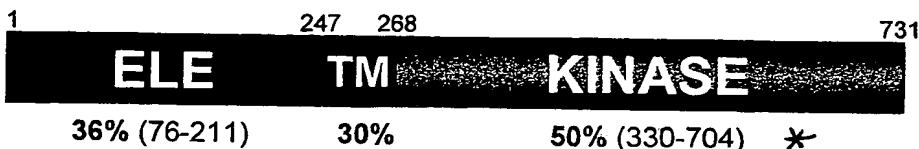


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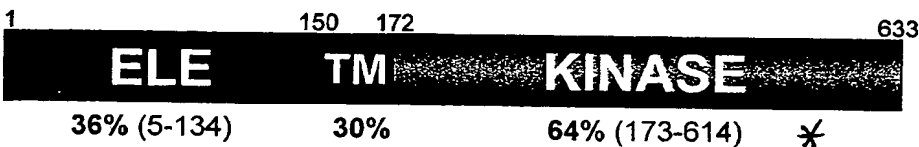
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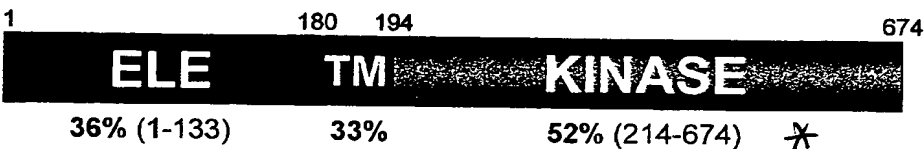
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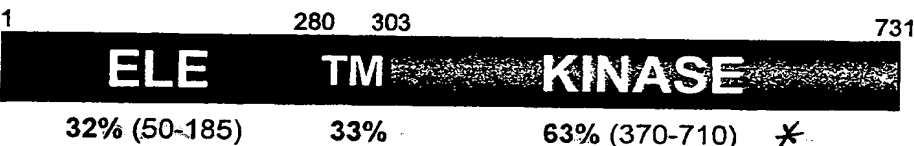
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†CAA18590



All have no signal peptide and predicted to be Type1b integral membrane proteins

† Predicted proteins from the Arabidopsis genome sequencing project.

ELE: Extensin-like extracellular ; TM: Transmembrane domain ; * Red = sequence identity to PERK1 domain

	PERK1	ACC98010	AAD15491	CAA18823	CAA18590	
PERK1		50%	64%	52%	63%	
ACC98010	36%		54%	50%	52%	Kinase Domains
AAD15491	36%	25%		63%	51%	
CAA18823	36%	29%	27%		60%	
CAA18590	32%	36%	30%	27%		
						ELE Domains

FIGURE 11

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